Respiratory Medicine (2006) **100**, 1724–1733

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Increased serum vascular endothelial growth factor levels in microscopic poly angiitis with pulmonary involvement

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Received 19 December 2005; accepted 7 February 2006

KEYWORDS
VEGF;
Composite physiologic index;
Neutrophil;
CD11b

Summary Microscopic polyangiitis (MPA) is a systemic necrotizing vasculitis that affects small vessels, resulting in a wide spectrum of organ involvement including the lungs. However, there are little serological markers that predict its prognosis or severity of pulmonary involvement. Vascular endothelial growth factor (VEGF) is an angiogenic mediator, which has been reported to be elevated in systemic vasculitis. In this study, we measured serum VEGF levels in 22 MPA patients with pulmonary involvement. We also investigated VEGF expression in pulmonary cells using flow cytometry analysis. We found that serum VEGF levels in MPA patients were significantly higher than those in respiratory or urinary tract infection. The serum VEGF levels decreased in parallel with the improvement of MPA symptoms. The serum VEGF levels in MPA patients who died within 5 years were significantly higher than those who survived more than 5 years. The sensitivity of VEGF levels to distinguish MPA patient with poor prognosis from those with good prognosis was 90.9%, and specificity was 81.8% (cutoff value = 802.5 pg/ml). The serum VEGF levels showed significant positive correlation with the composite physiological index, which indicates the severity of pulmonary lesion. In flow cytometry analysis, CD11b positive bronchoalveolar lavage fluid cells expressed VEGF. Immunohistochemically, alveolar macrophages, tissue infiltrating inflammatory cells and alveolar epithelial cells stained positive for VEGF. Measurement of serum VEGF levels in MPA might become one of the markers for prognosis and the severity of pulmonary involvement in MPA. VEGF might contribute to the development of pulmonary lesion of MPA.

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doi:10.1016/j.rmed.2006.02.006

Introduction

Microscopic polyangiitis (MPA) is a rare systemic disease that shows necrotizing vasculitis in small vessels, resulting in a wide spectrum of organ involvement including the lungs.[1] The etiology of this condition is unknown and the MPA patient typically presents with renal dysfunction or pulmonary hemorrhage[2] and its 5-year survival rate is reported as 45%.[3] In addition, several studies described that MPA reveals a variety of pulmonary findings.[4–6] However, there has been little serological marker for its prognosis or severity of pulmonary involvement.

Vascular endothelial growth factor (VEGF), a homodimeric, heparin-binding glycoprotein of 34–42 KD, is one of the major mediators of angiogenesis and vascular permeability.[7,8] VEGFs and their receptors have been implicated in the regulation of vascular permeability in many organ systems, including the lung.[9] There has been several reports describing the importance of VEGF in systemic vasculitis, such as Wegener's granulomatosis,[10] Giant cell arteritis[11] and Kawasaki disease.[12] In pulmonary inflammatory disorders, VEGF has been reported to play an important role in the development of their lesion via its capacity to increase vascular permeability.[9,13–15] In this study, we hypothesized that serum VEGF level might be useful to predict the prognosis of MPA and the severity of pulmonary involvement in MPA and investigated 22 MPA patients who have pulmonary involvement. We found that serum VEGF level of MPA patients had significant positive correlation with the severity of pulmonary lesion and activated pulmonary neutrophils expressed VEGF.

Materials and methods

Patients

This study was reviewed and approved by the Kagoshima University Faculty of Medicine Committee on Human Research. We investigated retrospectively 22 patients with MPA who were admitted to the Division of Respiratory Medicine, Respiratory and Stress Care Center, Kagoshima University Hospital from 1993 to 2005. There were 9 men and 13 women whose mean age was 68.0 ± 9.14 years old (mean $\pm$ standard deviation, median = 70 years old). For comparison, we also investigated 16 patients with respiratory tract infection (male: female = 7:9, mean $\pm$ standard deviation = 67.9 ± 10.3 years old, median = 68 years old, pneumonia = 10, acute bronchitis = 6), 13 patients with urinary tract infection (male : female = 5:8, mean $\pm$ standard deviation = 68.2 ± 11.1 years old, median = 67 years old). The MPA patients were diagnosed according to the definitions of the 1992 Chapel Hill Consensus Conference[16] based on typical history and characteristic clinical findings and confirmed by histology. Disease activity was assessed by using the standardized Birmingham Vasculitis Activity Score (BVAS).[17] This clinical index is based on symptoms and signs in nine categories: systemic signs; skin; mucous membranes and eyes; ear, nose and throat (ENT); chest; heart and vessels; gastrointestinal tract; kidney; and nervous system. We excluded patients with collagen vascular diseases including rheumatoid arthritis, diabetes mellitus, acute or chronic liver disease, and immunological abnormalities that predispose to opportunistic infection. We also excluded the MPA patients with respiratory tract infection and urinary tract infection. Peripheral blood analysis, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), KL-6, the smoking index (number of cigarettes smoked per day-smoking years) and arterial oxygen pressure (PaO_2) of all patients were also determined.

All MPA patients were positive for myeloperoxidase (MPO) anti-neutrophil cytoplasm autoantibodies (ANCA). All patients with respiratory tract infection and urinary tract infection were negative for MPO-ANCA. All MPA patients were treated with corticosteroids and immuno-suppressive drug (cyclophosphamide). In MPA, 5-year survival rate was 50% and mean survival period was 31.9 ± 28.1 months. Six patients died because of respiratory failure, 4 patients died because of infectious disease and one patient died because of disseminated intravascular coagulation.

Measurement of VEGF

We measured serum levels of VEGF before the patients underwent therapy of the patients with MPA, respiratory tract infection and urinary tract infection. In 15 patients with MPA, serum VEGF levels were determined before therapy and 3 months after the start of therapy. We also measured serum VEGF levels in 10 healthy volunteers (male:female = 4:6, mean age = 64.9 ± 12.3 years old). All participants gave written consent to participate in this study.

VEGF concentrations in sera or in bronchoalveolar lavage fluid (BALF) were measured in duplicate for each sample using a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) that recognizes the

soluble isoforms $VEGF_{121}$ and $VEGF_{165}$. This assay is sensitive to 9 pg/ml (0.2 pM) VEGF and does not cross-react with platelet-derived growth factor or other homologous cytokines. We also measured interleukin-12 (IL-12) concentrations in sera using ELISA kit that recognizes both IL-12, p40 and p70. This assay is sensitive to 7.5 pg/ml of IL-12 and does not cross-react with other homologous cytokines. Optical density at 450 nm was measured on a Titertek Multiskan MC plate reader (Flow Laboratories, Helsinki, Finland), and VEGF or IL-12 concentration was determined by linear regression from a standard curve using GraphPad software (San Diego, CA) for analysis.

Composite physiologic index

To evaluate the morphologic severity of pulmonary lesion of MPA patients, we employed composite physiologic index (CPI) which was introduced by Wells et al.[18] The formula for the CPI was as follows: CPI = 91.0 × (0.65 × percent predicted diffusing capacity for carbon monoxide [DL_{CO}])− (0.53 × percent predicted FVC)+(0.34 × percent predicted FEV_1).

Radiographic analysis

We examined the affected pulmonary segments on high-resolution computed tomography (HRCT) to evaluate the distribution of the lesion in patients with MPA. The thickness of each slice was 1 cm and 30 slices were examined in total. The radiographic findings of each subject were evaluated independently by two investigators (a pulmonologist and a radiologist) who have been blinded to the clinical data. Disagreements of two CT readers were reviewed jointly and resolved with consensus evaluation.

BALF analysis

A bronchofibrescope (Olympus BF type p20, Olympus Co., Tokyo, Japan) was wedged into the right B_4 segment of the lung in order to collect BALF cells from 11 MPA patients. Four 40-ml aliquots of sterile physiological saline were instilled at 37 °C, and recovered by gentle suction. The recovered fluid was immediately filtered through sterilized gauze and the lavage fluid was centrifuged in a cytometer (KN-70, Kubota Ltd., Tokyo, Japan) at 44*g* for 5 min and stained with May–Giemsa stain in order to identify cell populations. The supernatants were stored at −20 °C for the measurement of VEGF. Five hundred cells, excluding epithelial cells, were identified per slide in order to establish differential cell counts, and the counts were expressed in percentages. Concurrently, 1×10^5 cells were suspended in 50 μl of cold PBS containing 0.1% sodium azide, 10 ng/ml BSA and 20 μg/ml of human IgG and incubated for 10 min on ice. The cells were then incubated for an additional 15 min on ice with FITC-conjugated CD3 or CD18 monoclonal antibody (Becton Dickinson Co., Mountain View, CA, USA) in combination with mouse monoclonal anti-human CD25 or CD11b antibody (Becton Dickinson Co.). The cells were washed with PBS, and incubated with biotin-conjugated goat anti-mouse IgG antibody for 15 min on ice. Cells were again washed with PBS, and incubated with PE-conjugated streptavidin for 15 min on ice. At the end of the incubation period, 7AAD (PharMingen, San Diego, CA, USA) was added to each tube. The cells were washed with PBS, and subsequently analyzed by flow cytometry using a FACScan (Becton Dickinson). Dead cells, identified by the 7AAD incorporation, were gated out. Results were processed using the CellQuest software (Becton Dickson) as described previously.[19]

Intracellular staining for VEGF of BALF cells

We utilized an intracellular staining technique for VEGF in BALF cells as described previously.[20] BALF cells from 11 MPA patients were collected as described above. After washing with phosphate-buffered saline (PBS), BALF cells were separated to mononuclear cells and granulocytes using HISTOPAQUE 1077 and 1119 (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. Granulocytes were stained with PE-conjugated anti-CD11b (Becton Dickinson Co., Mountain View, CA, USA) and mononuclear cells were stained with CD25 monoclonal antibody (Becton Dickinson Co.). Cells were washed with PBS, resuspended in PBS containing 4% paraformaldehyde and then incubated at 4° for 20 min. After washing with PBS, cells were resuspended in PBS containing 1% saponin and incubated for 1 h at 4 °C with antibody for VEGF (goat polyclonal immunoglobulin IgG; A-20, Santa Cruz, CA, USA). Cells were rewashed with PBS containing 1% saponin and then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG antibody (Cedarlane, Ontario, Canada). After incubation, the cells were washed in PBS containing 1% saponin, resuspended in PBS and subsequently analyzed by flow cytometry using a FACScan (Becton Dickinson). Dead cells, determined by the incorporation of 7AAD (Becton Dickinson), were gated out. Results were processed using the CellQuest software (Becton Dickson).

Immunohistochemical staining for VEGF

Two MPA patients received lung biopsy via video-associated thoracoscopic surgery. Both biopsied regions showed ground-glass attenuation with consolidation on chest HRCT. Immunohistochemical staining for VEGF was performed using a rabbit polyclonal antibody (Santa Cruz, CA) employing the DAB method using the biopsied lung samples of these patients as described previously.[13] Briefly, 4 μm-thick sections were dewaxed and rehydrated. For optimal antigen retrieval, sections were pressure cooked in 0.01 M citrate buffer (pH 6.0) for 90 s. Endogenous peroxidase activity was blocked using a 3% hydrogen peroxide solution in methanol for 10 min. After washing, sections were incubated with primary antibody solution for 2 h at room temperature using a 1:150 concentration working dilution of the antibody. Negative control slides were incubated with rabbit polyclonal antibody (Super Sensitive Rabbit; Biogenex). After washing, secondary biotynated anti-immunoglobulin antibody (Biogenex) was added, and the mixture was incubated for 30 min at room temperature. The sections were again washed and streptavidin conjugated to horseradish peroxidase (Biogenex) was incubated for 30 min and then rinsed off with deionized water. DAB substrate solution was then added, and the mixture was incubated for 10 min. A brown color reaction represented a positive result.

Statistical analysis

We used one-way factorial analysis of variance (ANOVA) with the Bonferroni–Dunn test, Whitney–Mann test and Wilcoxon signed-rank test. For statistical analysis, Stat view and MedCalc software were used. A *P*-value below 0.05 was considered significant. Most values were expressed as mean ± standard deviation (SD).

Results

Table 1 shows the clinical features of MPA patients. All MPA patients complained fever and hematuria. Four patients did not complain any respiratory symptoms but had arthralgia or dermatitis. Eighteen patients complained cough and most cases were not productive. Table 2 shows the chest HRCT findings of MPA patients. Eighteen patients had mixed radiographic findings and all of them complained respiratory symptoms. The most frequent chest HRCT finding was ''Traction bronchiectasis'' and the next was ''Ground-glass attenuation''. Fifty percent of the patients showed ''Honeycombing''. Fourteen patients had mixed radiographic findings and these patients complained dyspnea. In BALF analysis, total cell count was $1.75 \pm 0.88 \times 10^5$/ml, the number of macrophages was $1.24 \pm 0.23 \times 10^5$/ml ($71.2 \pm 6.24\%$), lymphocytes was $0.23 \pm 0.07 \times 10^5$/ml ($13.1 \pm 5.1\%$), neutrophils was $0.25 \pm 0.04 \times 10^5$/ml ($14.4 \pm 4.46\%$), and eosinophils was $0.02 \pm 0.01 \times 10^5$/ml ($1.36 \pm 0.51\%$). The percentage of CD25+/CD3+ cells was $44.4 \pm 13.6\%$ and the percentage of CD11b+/CD18+ cells was $52.9 \pm 10.4\%$.

The serum VEGF levels in patients with MPA were significantly higher than in patients with respiratory tract infection (pneumonia and acute bronchitis), urinary tract infection or healthy volunteers (MPA patients: mean ± SD = 1104.5 ± 684.3 pg/ml; patients with respiratory tract infection: mean ± SD = 391.2 ± 133.8 pg/ml; patients with urinary tract

Table 1 Clinical features of MPA patients on admission.

Age (years old)	68.0 ± 9.14 (70)*	Laboratory data	
Male:female	9:13	WBC	10915 ± 3167/μl
Symptoms		Neu.	79 ± 9.22%
Fever (+)	22/22	Lym.	14 ± 7.02%
Weight loss (+)	2/22	Hb.	11 ± 1.85g/dl
Cough (+)	18/22	CRP	10.2 ± 4.46 mg/dl
Sputum (+)	5/22	ESR	63 ± 23.7 mm/h
Dyspnea (+)	14/22	KL-6	607 ± 394.1U/ml
Arthralgia (+)	6/22	Cr.	1.00 ± 0.58 mg/dl
Myalgia (+)	4/22	Hematuria (+)	22/22
Dermatitis (+)	3/22	Albuminuria (+)	14/22
Neuropathy (+)	8/22	MPO-ANCA	338 ± 512.2EU

*Median value in parenthesis, WBC: white blood cell, Neu.: neutrophil, Lym.: Lymphocytes, Hb.: hemoglobin, Plt.: platelet, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, Cr.: creatinine, MPO-ANCA: myeloperoxidase-antineutrophil cytoplasmic antibody.

Table 2 Findings on chest CT in 22 MPA patients.

Finding	No. of patients (%)
Ground-glass attenuation	14 (63.6)
Consolidation	9 (40.9)
Thickening of bronchovascular bundle	9 (40.9)
Nodules	6 (27.3)
Honeycombing	11 (50)
Traction bronchiectasis	19 (86.4)
Pleural effusion	7 (31.8)
Lymph node swelling	3 (13.6)

infection: mean $\pm$ SD = 364.1 $\pm$ 118.9 pg/ml; healthy volunteers: mean $\pm$ SD = 98.3 $\pm$ 43.3 pg/ml; Fig. 1a). The sensitivity of VEGF levels to distinguish MPA from respiratory tract infection or urinary tract infection was 81.8%, and specificity was 93.5% (cutoff value = 527.5 pg/ml, positive predicted value = 90%, negative predicted value = 87.9%). The serum VEGF levels of 15 patients with MPA significantly decreased 3 month after the start of therapy (before = 1084.7 $\pm$ 345.9 pg/ml, after = 339.1 $\pm$ 156.9 pg/ml, $P<0.05$, Whitney–Mann test, Fig. 1b). Three months after the start of therapy, the clinical symptoms and serum inflammatory markers such as CRP of MPA patients were improved successfully by the treatment. The serum VEGF levels in MPA patients with poor prognosis (died within 5 years, 1491.3 $\pm$ 705.9 pg/ml) was significantly higher than those with good prognosis (survived more than 5 years, 786.1 $\pm$ 511.9 pg/ml, $P<0.05$, Whitney–Mann test). The sensitivity of VEGF levels to distinguish MPA patient who died within 5 years from MPA patients who survived more than 5 years was 90.9%, and specificity was 81.8% (cut off value = 802.5 pg/ml, positive predicted value = 83.3%, negative predicted value = 90%). The serum VEGF levels did not show significant correlation with MPO-ANCA level ($r = 0.136$, $P = 0.5$), platelet counts ($r = 0.323$, $P = 0.1$), CRP level ($r = 0.111$, $P = 0.9$), ESR ($r = 0.141$, $P = 0.8$), KL-6 ($r = 0.071$, $P = 0.7$), smoking index ($r = 0.139$, $P = 0.3$) and PaO_2 level ($r = -0.338$, $P = 0.08$). Regarding IL-12, serum IL-12 levels in healthy volunteers was significantly lower than those of other three groups ($P<0.01$, Bonferroni–Dunn with one way factorial ANOVA, MPA patients: mean $\pm$ SD = 194.5 $\pm$ 114.8 pg/ml; patients with respiratory tract infection: mean $\pm$ SD = 206.1 $\pm$ 106.7 pg/ml; patients with urinary tract infection: mean $\pm$ SD = 182.3 $\pm$ 112.7 pg/ml; healthy volunteers: mean $\pm$ SD = 57.9 $\pm$ 35.4 pg/ml). However, there was no significant difference of serum IL-12 levels among the MPA patients, patients with respiratory tract infection and patients with urinary tract infection. Also, there was no significant difference of serum IL-12 levels between the MPA patient with poor prognosis (MPA patients who died within 5 years, 211.3 $\pm$ 121.3 pg/ml) and the MPA patients with good prognosis (MPA patients who survived more than 5 years, 177.8 $\pm$ 111.1 pg/ml). The serum IL-12 levels did not show significant correlation with CPI ($r = 0.193$, $P = 0.395$).

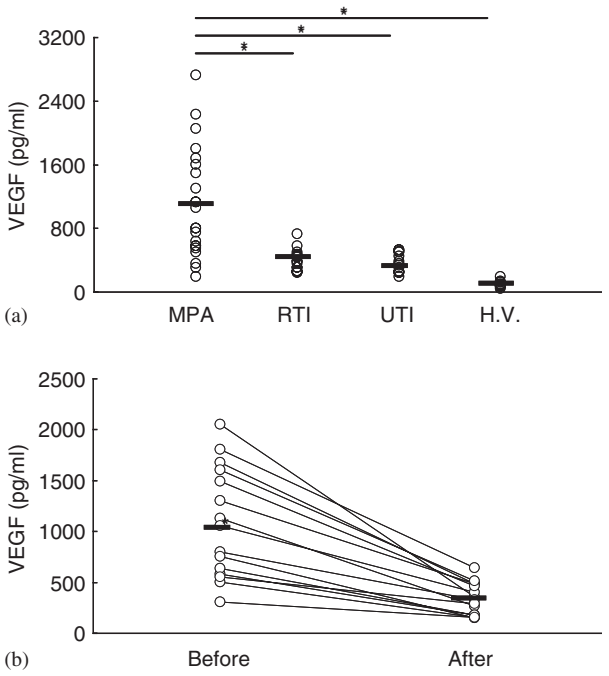

Figure 1 Serum VEGF levels in each patient group. The serum VEGF level in MPA patients was significantly higher than those in patients with respiratory tract infection, urinary tract infection and healthy volunteers (a, *$P<0.01$, Bonferroni–Dunn test with one-way factorial ANOVA, MPA: microscopic polyangiitis, RTI: respiratory tract infection, UTI: urinary tract infection, HV: healthy volunteer). The serum VEGF levels significantly decreased 3 months after the start of therapy (b, $P<0.05$, Whitney–Mann test).

Serum VEGF levels in MPA patients showed significant positive correlation with CPI ($r = 0.801$, $P<0.0001$, Wilcoxon signed-rank test, Fig. 2a) and BVAS ($r = 0.583$, $P<0.05$, Wilcoxon signed-rank test, Fig. 2b). Serum MPO-ANCA levels did not show any significant correlation with visual score ($r = 0.109$, $P = 0.12$) and BVAS ($r = 0.139$, $P = 0.1$). In MPA, serum VEGF levels of the patients who showed ground-glass attenuation (GGA) in chest CT were significantly higher than the patients without ground-glass attenuation (GGA+ = 1368.7 $\pm$ 705.9 pg/ml, GGA− = 723.1 $\pm$ 402.5 pg/ml, $P<0.05$, Whitney–Mann test, Fig. 3a). Also, serum VEGF levels of the patients who showed

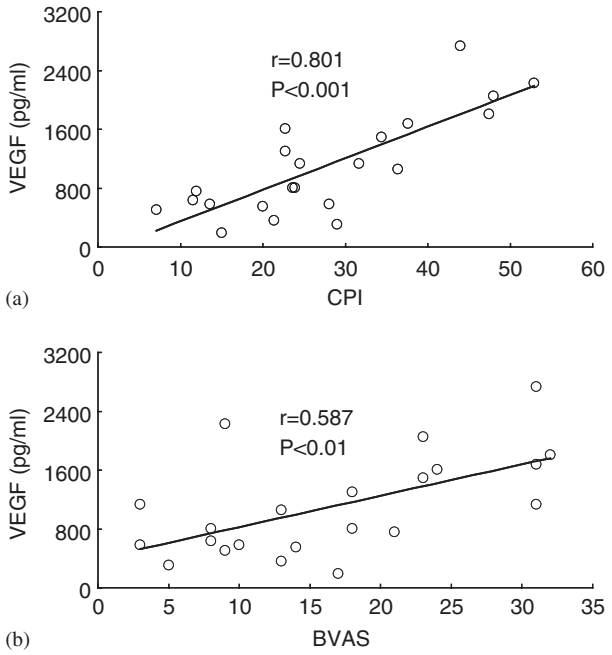

Figure 2 Correlation between serum VEGF level and visual score or BVAS. The serum VEGF levels in MPA patients showed significant correlation with visual score (a, $r = 0.809$, $P < 0.001$, Wilcoxon signed-rank test) and BVAS (b, $r = 0.587$, $P < 0.01$, Wilcoxon signed-rank test).

consolidation in chest CT were significantly higher than the patients without consolidation (consolidation+ $= 1523.6 \pm 681.3$ pg/ml, consolidation− $= 685.5 \pm 364.7$ pg/ml, $P < 0.01$, Whitney–Mann test, Fig. 3b). Concerning the other chest CT appearance such as honeycombing (Fig. 3c), there was no significant difference of serum VEGF levels between the patients with or without chest CT appearance.

Concerning the intracellular staining for VEGF, CD11b+ pulmonary granulocytes (Fig. 4a), not CD25+ pulmonary mononuclear cells (Fig. 4b), were stained positive for VEGF. The VEGF+/CD11b+ granulocyte percentage showed significant positive correlation with CD11b+/CD18+ BALF cell percentage (Fig. 4c). The VEGF+/CD11b+ granulocyte percentage also showed significant positive correlation with CPI (Fig. 4d). The CD11b+/CD18+ BALF cell percentage showed significant positive correlation with serum VEGF levels ($r = 0.721$, $P < 0.05$). The VEGF+/CD11b+ BALF cell percentage showed positive correlation with serum VEGF levels, however it was not statistical significant ($r = 0.511$, $P = 0.052$). The VEGF levels in BALF showed significant positive correlation with CPI ($r = 0.812$, $P < 0.01$) and with VEGF+/CD11b+ BALF cell percentage ($r = 0.629$, $P < 0.05$).

In immunohistochemical analysis, infiltrating inflammatory cells, alveolar macrophages and alveolar epithelial cells were stained positive for VEGF (Fig. 5).

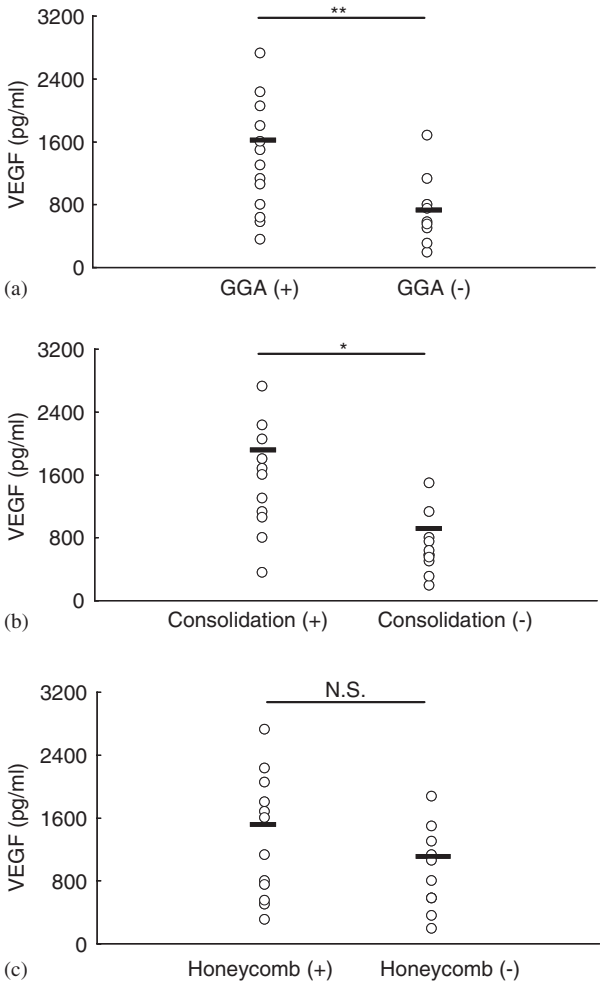

Figure 3 Comparison of serum VEGF level in each chest CT appearance. The serum VEGF levels in patients with ground-glass attenuation (a, $P < 0.05$, Whitney–Mann test, GGA: ground-glass attenuation) or consolidation (b, $P < 0.01$, Whitney–Mann test) were significantly higher than those without these shadows. There was no significant difference of serum VEGF levels between the patients with honeycombing and those without honeycombing (c).

Discussion

In this study, the serum VEGF levels of MPA patients with poor prognosis were significantly higher than those with good prognosis. Five-year survival rate in our study was 50% and this is almost same with previous reports.[3,21] The sensitivity and specificity of VEGF levels for the prediction of poor prognosis were 90.9% and 81.8%, respectively (cut off

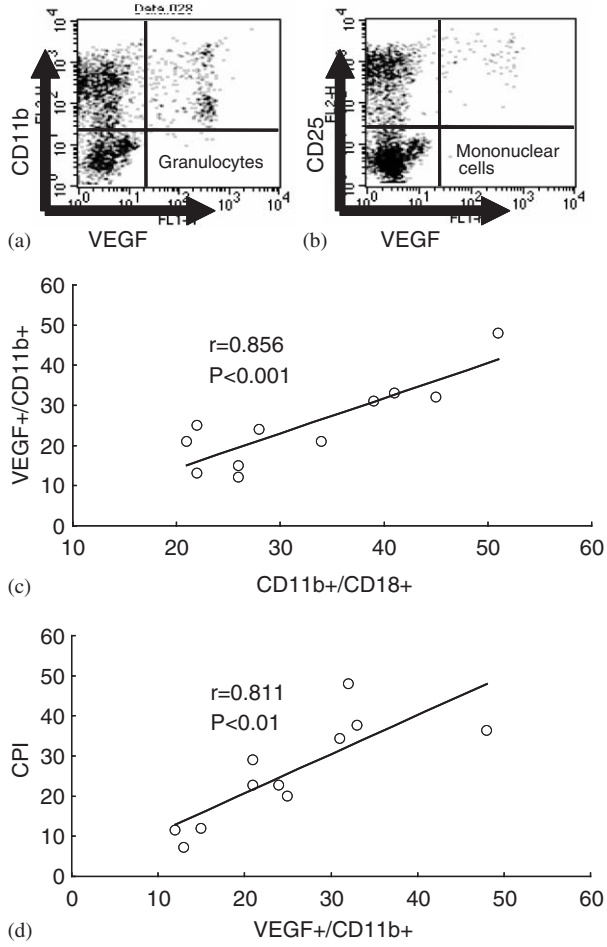

Figure 4 Analysis of VEGF expression in bronchoalveolar lavage fluid (BALF) cells. CD11b+ pulmonary granulocytes expressed high amount of VEGF (a, representative data in 11 cases), however CD25+ pulmonary mononuclear cells expressed little amount of VEGF (b, representative data in 11 cases). The CD11b+/CD18+ percentage showed significant positive correlation with VEGF+/CD11b+ BALF cells (c, $r = 0.856$, $P < 0.001$, Wilcoxon signed-rank test) and visual score (d, $r = 0.813$, $P < 0.01$, Wilcoxon signed-rank test).

value = 802.5 pg/ml). VEGF may be a useful screening marker to predict poor prognosis of MPA, as a negative result greatly reduces the death within 5 years. However, a cautious follow up for the MPA patients with low serum VEGF levels is also necessary as the specificity is relatively low. In our study, the serum VEGF levels showed significant positive correlation with disease activity. Hypoxia[22] and smoking[23] that can affect serum VEGF levels did not show significant correlation with serum VEGF levels. There has been several reports showing the clinical value of VEGF in monitoring disease activity in vasculitis including MPA.[24–26] Our result is compatible with previous reports and we propose that serum VEGF might become one of the serum markers that reflect disease activity of MPA. We also showed that serum VEGF levels in MPA patients were significantly higher than those in respiratory tract infection and urinary tract infection. However, there some overlap between the VEGF levels of the MPA and respiratory tract infection or urinary tract infection patients in our study. This fact indicates that measurement of VEGF alone cannot be a definitive marker to distinguish MPA from respiratory or urinary traction infection and suggest the necessity of measurement of standard markers such as MPO-ANCA for the accurate diagnosis and for the prediction of exact prognosis.

The MPA cases investigated in this study showed various chest HRCT appearance. In MPA, it was traditionally considered that the predominant pulmonary symptom is hemoptysis and this symptom reflects the alveolar hemorrhage caused by capillaritis.[1] Therefore, the traditionally reported chest radiographic feature was patchy, bilateral airspace opacities caused by alveolar hemorrhage.[1,6] However, resent studies found that MPA reveals a variety of pulmonary findings, including ground-glass attenuation, consolidation, thickening of bronchovascular bundles, and honeycombing.[4,5] Especially, Ando et al.[4] reported that the chest HRCT findings consisted of ground-glass attenuation in 94% of the patients, consolidation in 78%, and thickening of bronchovascular bundles in 51%. In our study, the chest HRCT findings consisted of traction bronchiectasis in 86.4%, ground-glass attenuation in 63.6%, and honeycombing in 50% of the MPA patients. Thus, the chest HRCT appearance of MPA is variable and, therefore, we believe that the patients with pulmonary shadows as described above should be examined about the existence of ANCA.

In our study, the serum VEGF level was significantly higher in patients with ground-glass attenuation or consolidation than those without these shadows. In addition, serum VEGF levels showed significant positive correlation with the affected pulmonary area and disease activity. In MPA, it was reported that the pathological feature of ground-glass attenuation is chronic inflammation with vasculitis and the pathological feature of consolidation is alveolar hemorrhage.[4] Taken together, it might be possible that these shadows reveal the existence of active pulmonary lesion in MPA patients. Regarding other shadows, Eschun et al. reported that pulmonary fibrosis shadow including honeycombing and traction bronchiectasis occur as a pulmonary manifestation of MPA and suggested that repeated episodes of alveolar hemorrhage

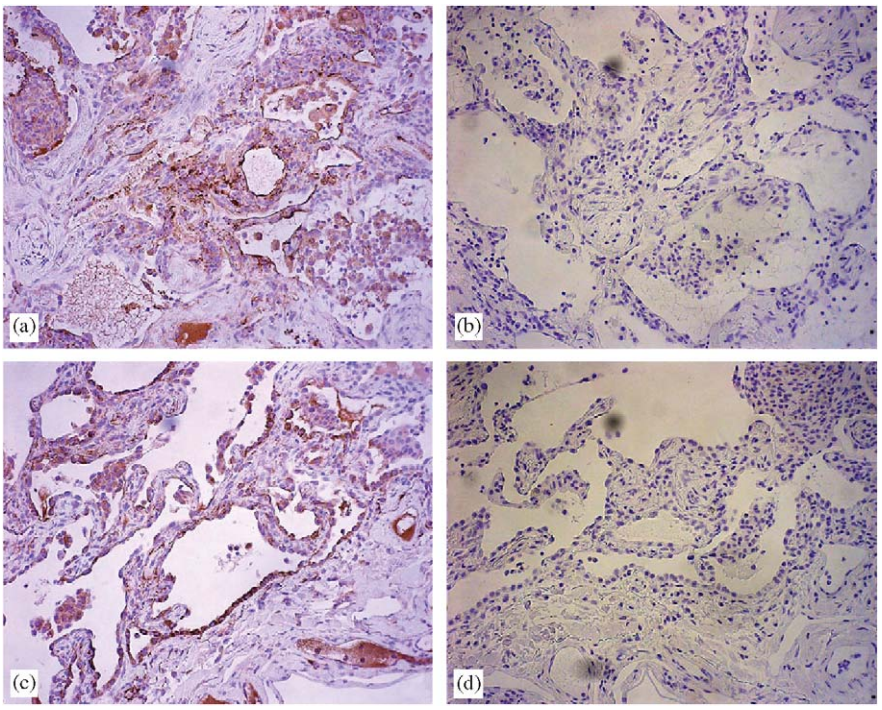

Figure 5 Immunohistochemical analysis for VEGF. Infiltrating inflammatory cells, alveolar macrophages and alveolar epithelial cells were stained positive for VEGF (a and c: stained with anti-VEGF antibody, b and d: negative control. $\times$ 400 original magnification).

could be the forerunner of interstitial fibrosis in MPA.[6] In our study, there was no significant difference of serum VEGF levels between the patients with traction bronchiectasis or honeycombing and without these shadows. It might be possible that these shadows (traction bronchiectasis or honeycombing) were the result of repeated pulmonary capillaritis and, therefore, no significant difference of the serum VEGF level was seen. We propose the necessity of further studies to clarify the features of chest radiographic appearance of MPA.

Our study showed that CD11b+ pulmonary granulocytes expressed VEGF. The VEGF+/CD11b+ granulocyte percentage showed significant positive correlation with CD11b+/CD18+ BALF cell percentage. The CD18 (β_2-integrin) is heterodimeric integral membrane glycoprotein expressed mainly on neutrophils and consist of a common CD18 β-chain that can associate with one of four α-chains termed CD11a (LFA-1), CD11b (Mac-1), CD11c (p150), and CD11d.[27] In experiments with blocking antibodies specific for β_2-integrin α-chains, it was determined that neutrophil adhesion to epithelial cells is mediated exclusively by CD11b and CD18.[28] CD11b (Mac-1) is the receptor for complement 3bi[29] and it contributes to the neutrophil rolling on endothelium and facilitating phagocytosis of opsonized particles in neutrophils.[30] Therefore, we think CD11b+ pulmonary granulocytes are activated pulmonary neutrophils that can adhere and roll on endothelium. Proteinase-3 and MPO-ANCA can activate neutrophils via Fc-gamma receptor IIa-mediated mechanism.[31] Neutrophils are able to express VEGF[32,33] and, especially, neutrophil-derived VEGF is reported to play a role in regulating early vascular responses in Kawasaki disease.[33] Tumor necrosis factor alpha, which plays an important role in MPA,[34] can up-regulate the expression of VGEF.[35] In contrast, CD25+ pulmonary mononuclear cells, which are considered as activated lymphocytes,[36] did not express VEGF in our study. CD11b+ pulmonary granulocytes showed significant positive correlation with serum VEGF levels. BALF VEGF levels showed significant correlation with VEGF+/CD11b+ cell percentage. In immunohistochemical analysis, small infiltrating inflammatory cells were stained positive for VEGF. Taken together, we think that pulmonary activated neutrophils might be one of the cellular sources of VEGF in MPA.

In our study, serum IL-12 levels, one of the anti-angiogenic cytokines,[37,38] did not show any correlation with MPA prognosis and severity of pulmonary lesion in our study. Furthermore, in immunohistochemical analysis, not only inflammatory cells but

also alveolar macrophages and alveolar epithelial cells were stained positive for VEGF. Alveolar macrophages[13] and alveolar epithelial cells[39,40] are capable to produce VEGF. The serum VEGF levels of MPA patients decreased after the treatment with corticosteroids and cyclophosphamide. We think these treatments might contribute to the decrease of serum VEGF because corticosteroids can inhibit VEGF production from pulmonary cells[41,42] and cyclophosphamide can also inhibit VEGF production in vivo.[43] Indeed, VEGF induce vascular endothelial cell proliferation and cause intense angiogenesis[44] and increase vascular permeability.[7,8] In our study, BALF VEGF levels showed significant correlation with CPI. VEGF production in the pulmonary lesion might contribute to the development of pulmonary involvement in MPA. Further studies addressing this point might be important to clarify the pathogenesis of pulmonary involvement in MPA.

Acknowledgement

We wish special thanks to Mrs. Rumi Matsuyama for her excellent help.

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